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#2

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Signed A M Brewer.

Dated 18 September 2000

official use

10 SEP 1999

13SEP99 E476083-1 D03312
P01/7700 0.00 - 9921477.7

Your reference 230P81229

10 SEP 1999

9921477.7

Notes
Please type, or write in dark ink using CAPITAL letters. A prescribed fee is payable for a request for grant of a patent. For details, please contact the Patent Office (telephone 071–438 4700).

Rule 16 of the Patents Rules 1990 is the main rule governing the completion and filing of this form.

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The Patent Office

Request for grant of a Patent

Form 1/77

Patents Act 1977

❶ Title of invention

1 Please give the title of the invention: NEW ACTIVE MARINE ALKALOIDS

❷ Applicant's details

☐ **First or only applicant**

2a If you are applying as a corporate body please give:

Corporate name: Instituto Biomar S.A.

Country *(and State of incorporation, if appropriate)*: SPAIN

2b If you are applying as an individual or one of a partnership please give in full:

Surname

Forenames

2c **In all cases**, please give the following details:

Address: Polígono Industrial, Edificio CEI
Mod. 2.02 y 2.03
24231-Onzonilla (LEON)
SPAIN

UK postcode *(if applicable)*

Country

ADP number *(if known)* 7362368003 ES

2d, 2e and 2f: *If there are further applicants please provide details on a separate sheet of paper.*

Second applicant ***(if any)***

2d If you are applying as a corporate body please give:

Corporate name

Country *(and State of incorporation, if appropriate)*

2e If you are applying as an individual or one of a partnership please give in full:

Surname

Forenames

2f **In all cases**, please give the following details:

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❸ *An address for service in the United Kingdom must be supplied*

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❸ Address for service details

3a Have you appointed an agent to deal with your application?

Yes [X] No [ ] ➡ ***go to 3b***

⬇ ***please give details below***

Agent's name: Marks & Clerk

Agent's address: 57-60 Lincoln's Inn Fields
LONDON
WC2A 3LS.

Postcode

Agent's ADP number: 18001

3b: *If you have appointed an agent, all correspondence concerning your application will be sent to the agent's United Kingdom address.*

3b If you have not appointed an agent please give a name and address in the United Kingdom to which all correspondence will be sent:

Name

Address

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Daytime telephone number *(if available)*

ADP number *(if known)*

❹ Refer nce number

4 Agent's or applicant's reference number *(if applicable)* 230P81229

❺ Claiming an earli r application date

5 Are you claiming that this application be treated as having been filed on the date of filing of an earlier application?

Please mark correct box

Yes ☐ No ☒ ➡ ***go to 6***

⬇

please give details below

❑ number of earlier application or patent number

❑ filing date

(day month year)

❑ and the Section of the Patents Act 1977 under which you are claiming:

Please mark correct box

15(4) (Divisional) ☐ 8(3) ☐ 12(6) ☐ 37(4) ☐

❻ Declaration of priority

❻ If you are declaring priority from a PCT Application please enter 'PCT' as the country and enter the country code (for example, GB) as part of the application number.

Please give the date in all number format, for example, 31/05/90 for 31 May 1990.

6 If you are declaring priority from previous application(s), please give:

Country of filing	Priority application number *(if known)*	Filing date *(day, month, year)*

❼ *The answer must be 'No' if: any applicant is not an inventor there is an inventor who is not an applicant, **or** any applicant is a corporate body.*

❼ Inventorship

7 Are you (the applicant or applicants) the sole inventor or the joint inventors?

Please mark correct box

Yes ☐ No ☒ ➡ ***A Statement of Inventorship on Patents Form 7/77 will need to be filed (see Rule 15).***

❽ *Please supply duplicates of claim(s), abstract, description and drawing(s).*

❽ Checklist

8a Please fill in the number of sheets for each of the following types of document contained in this application.

Continuation sheets for this Patents Form 1/77	—		
Claim(s)	—	Description	5
Abstract	—	Drawing(s)	—

8b Which of the following documents also accompanies the application?

Please mark correct box(es)

Priority documents *(please state how many)*	—
Translation(s) of Priority documents *(please state how many)*	—
Patents Form 7/77 – Statement of Inventorship and Right to Grant *(please state how many)*	—
Patents Form 9/77 – Preliminary Examination/Search	—
Patents Form 10/77 – Request for Substantive Examination	—

❾ *You or your appointed agent (see Rule 90 of the Patents Rules 1990) must sign this request.*

❾ Request

I/We request the grant of a patent on the basis of this application.

Please sign here ➡ Signed Marks & Clerk Date 10 - 9 - 99 (day month year)

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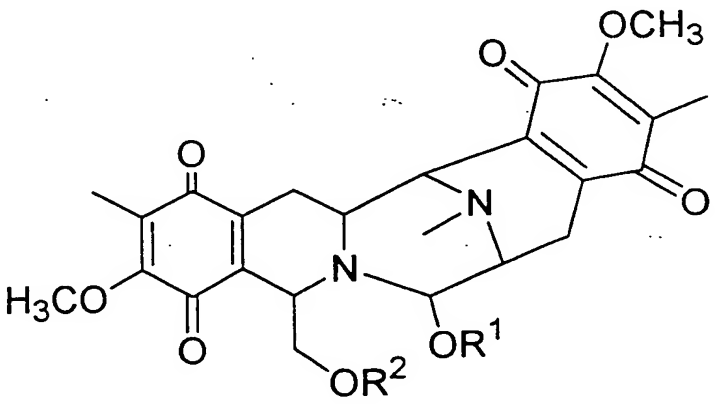
NEW ACTIVE MARINE ALKALOIDS

The present invention relates to a new active alkaloids isolated from the mollusc *Jorunna funebris*.

Summary of the Invention

Marine organisms, especially soft corals, sponges and tunicates, provide many secondary metabolites and exhibit a varying degree of biological activity (Faulkner, D.J. *Nat.Prod.Reports.*, **1999**, *16*, 155-198 and references cited therein).

The present invention provides new alkaloids having the following formula (I):



(I)

wherein $\mathbf{R^1}$ is selected from the group consisting of hydrogen, lower alkyl group and lower alkoxy group and $\mathbf{R^2}$ is selected from the group consisting of hydrogen and lower alkoxy.
In the definitions of the groups in formula (I), the lower alkyl and the lower alkyl moiety of the lower alkoxy mean a straight-chain or branched alkyl group having 1 to 6 carbon atoms such as methyl, ethyl, propyl, isopropyl, butyl, isobutyl, sec-butyl, tert-butyl, pentyl, neopentyl and hexyl.

More particularly, the present invention relates to Jorumycin (R^1=H and R^2=Ac in formula (I)), extracted and isolated from the mollusc *Jorunna funebris*.

Jorumycin exhibits antitumor activity. In particular, Jorumycin exhibits antitumor activity against cell lines derived from human solid tumors, such as human lung carcinoma, human colon carcinoma and human melanoma, and, the like, it is active against other tumor cell lines, like leukemia and lymphoma.

The present invention also provides a method of treating a mammal affected by a malignant tumor sensitive to a compound with the formula (I), which comprises administering a therapeutically effective amount of the compound with the formula (I), or a pharmaceutical composition thereof.

The present invention further provides pharmaceutical compositions which contain as active ingredient a compound with the formula (I), as well as a process for its preparation.

A further aspect of the invention is a method for preparing the compound Jorumycin (R^1=H and R^2=Ac in the formula (I)), which comprises extraction and isolation from the mollusc *Jorunna funebris*.

Preferred Embodiments of the Invention

Examples of pharmaceutical compositions include any solid (tablets, pills, capsules, granules, etc.) or liquid (solutions, suspensions or emulsions) with suitable formulation of oral, topical or parenteral administration, and they may contain the pure compound or in combination with any carrier or other pharmacologically active compounds. These compositions may need to be sterile when administered parenterally.

The correct dosage of a pharmaceutical composition comprising compounds with the formula (I), will vary according to the pharmaceutical formulation, the mode of application, and the particular situs, host and tumor being treated. Other factors like age, body weight, sex, diet, time of administration, rate of excretion, condition of the host, drug combinations, reaction

sensitivities and severity of the disease shall be taken into account. Administration can be carried out continuously or periodically within the maximum tolerated dose.

Antitumour Activity

Cells were maintained in logarithmic phase of growth in Eagle's Minimum Essential Medium, with Earle's Balanced Salts, with 2.0 mM L-glutamine, with non-essential amino acids, without sodium bicarbonate (EMEM/neaa); supplemented with 10% Fetal Calf Serum (FCS), 10^{-2} M sodium bicarbonate and 0,1 g/l penicillin-G + streptomycin sulfate.

A screening procedure has been carried out to determine and compare the antitumor activity of these compounds, using an adapted form of the method described by Bergeron et al. (Raymond J. Bergeron, Paul F. Cavanaugh, Jr., Steven J. Kline, Robert G. Hughes, Jr., Gary T. Elliot and Carl W. Porter. Antineoplastic and antiherpetic activity of spermidine catecholamide iron chelators. *Biochem. Bioph. Res. Comm.* **1984**, *121*, 848-854). The antitumor cells employed were P-388 (suspension culture of a lymphoid neoplasm from DBA/2 mouse), A-549 (monolayer culture of a human lung carcinoma), HT-29 (monolayer culture of a human colon carcinoma) and MEL-28 (monolayer culture of a human melanoma).

P-388 cells were seeded into 16 mm wells at $1x10^4$ cells per well in 1 ml aliquots of MEM 5FCS containing the indicated concentration of drug. A separate set of cultures without drug was seeded as control growth to ensure that cells remained in exponential phase of growth. All determinations were carried out in duplicate. After three days of incubation at 37°C, 10% CO_2 in a 98% humid atmosphere, an approximately IC_{50} was determined by comparing the growth in wells with drug to the growth in wells control.

A-549, HT-29 and MEL-28 cells were seeded into 16 mm wells at $2x10^4$ cells per well in 1 ml aliquots of MEM 10FCS containing the indicated concentration of drug. A separate set of cultures without drug was seeded as control growth to ensure that cells remained in exponential phase of growth. All determinations were carried out in duplicate. After three days of incubation at 37°C, 10% CO_2 in a 98% humid atmosphere, the wells were stained with 0.1%

Crystal Violet. An approximately IC_{50} was determined by comparing the growth in wells with drug to the growth in wells control.

The results are given in the following table:

	IC_{50} (μM)			
	P-388	**A-549**	**HT-29**	**MEL-28**
Jorumycin	0.02	0.02	0.02	0.02

Antitumour Activity

Jorumycin showed also activity against Gram-positive bacteria (Staphyloloccus and others).

Extraction and Isolation

Jorumycin has been isolated from the mollusc *Jorunna funebris* (Mollusca: Nudibranchia: Doridina: Kentrodorididae) collected off Mandapam (India) in April 1998. The product is present in the extract of both the mucus and mantle of the nudibranch. In a typical procedure, the frozen biological sample (mucus or animal body) was extracted with acetone. After removing the organic solvent at reduced pressure the residue was partitioned between water (15 mL) and ethyl acetate. The water phase was extracted for three times with ethyl acetate (on the whole 34 mL). The oily residue obtained by removing of the organic solvent at reduced pressure was fractionated by Sephadex LH-20 chromatography following the elution of the extract components by SiO_2-TLC (CHC_{l3}/MeOH 95:5, Rf = 0.4). The final purification of Jorumycin was obtained by sequential SiO2 chromatographies ($CHCl_3$/MeOH) and HPLC (Sherisorb S5W analytical column, isocratic elution with:n-hexane/CHC_{l3}/TEA 90:10:10, detector: Waters R401

differential refractometer). The product is soluble in CHC_{13}, CH_2Cl_2, MeOH. It is highly unstable in acid and basic media.

Jorumycin: ($C_{27}H_{30}N_2O_9$); $[\alpha]_D$= -57° (*c* 0.05 $CHCl_3$); IR (liquid film) 3310, 1731, 1700 cm^{-1}; UV (MeOH) 268 nm (ε=15000); ^{1}H-NMR ($CDCl_3$) δ 1.25 (m, 1H), 1.75 (s, 3H), 1.93 (s, 3H), 1.96 (s, 3H), 2.24 (d, 1H, J= 20.1 Hz), 2.26 (s, 3H), 2.65 (dd, 1H, J= 20.1 and 7.3 Hz), 2.84 (dd, 1H, J= 16.7 and 2.1 Hz), 3.16 (bdt, 1H, J= 12.0, 2.7 and 2.7 Hz), 3.18 (bs, 1H), 3.82 (dd, 1H, J= 11.2 and 3.5 Hz), 3.90 (bs, 1H), 3.98 (s, 3H), 4.00 (bs, 3H), 4.36 (bm, 1H), 4.40 (bs, 1H), 4.42 (dd, 1H, J= 11.2 and 3.7 Hz); ^{13}C-NMR ($CDCl_3$) δ 185.8 (s), 181.3 (s), 170.0 (s), 155.7 (s), 155.0 (s), 141.9 (s), 141.7 (s), 137.2 (s), 128.8 (s), 128.4 (s), 113.8 (s), 109.4 (s), 83.0 (d), 64.2 (t), 61.0 (d), 57.7 (d), 56.1 (d), 54.4 (d), 52.6 (d), 41.3 (q), 25.5 (t), 20.7 (q), 20.6 (t), 8.8 (q), 8.7 (q); ESMS (*m/z*) 526 (20, M+), 508 (100, M-H_2O), 494 (10, M-32). HRESMS (*m/z*) 508.189 (Δ + 5 mmu).

The present invention also provides use of the compound of formula 1 in the preparation of a medicament for the treatment or prophalaxis of tumours.

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